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Methods

An enhanced qPCR method for rapid *Agrilus planipennis* detection and monitoring

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Abstract

Emerald ash borer (EAB; *Agrilus planipennis*) represents a serious threat to North American and European ash species (*Fraxinus* spp.). Spread of EAB westwards, from European Russia and Eastern Ukraine, could lead to dramatic consequences for native European ash populations. Early detection is essential for fast and successful eradication of new populations. In this study, we developed a new TaqMan qPCR assay allowing for sensitive and specific detection of EAB. We tested the specificity of the assay against 17 European *Agrilus* spp., eight buprestid species and nine species belonging to other wood-associated beetle taxa. The qPCR assay provided reliable amplification from samples with DNA concentrations as low as 0.5 picograms per reaction. Moreover, DNA could be amplified from different sample types, such as egg casings, leaves, faeces and bore dust from larval galleries. Robustness of the assay was verified by performing a blind test with four different laboratories. Here we provide a highly specific, robust and sensitive assay which can be used for enhanced surveillance of *Agrilus planipennis* on the European continent.

Key words: *Agrilus planipennis*, Emerald Ash Borer, EAB, invasive forest insects, TaqMan qPCR, surveillance

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Introduction

Native to Far-East Asia, emerald ash borer *Agrilus planipennis* (Fairmaire, 1888) is a highly destructive insect species when introduced in a non-native habitat. *Agrilus planipennis* also known by the acronym EAB, is a host-specialized buprestid, that attacks primarily ash trees (*Fraxinus* spp.) (Baranchikov et al. 2014; Jendek and Poláková 2014; Herms 2015). Infested trees typically succumb within one to four years; however, in outbreak situations, mortality may occur within one or two years (Haack et al. 2002).

The life cycle of EAB typically spans one to two years, with flight activity occurring from mid-May to July. Females lay 70 to 100 eggs in bark cracks and crevices on ash trees. Once hatched, neonates penetrate the outer bark and tunnel

between the phloem and cambium layers. Larvae then overwinter in a pre-pupal stage before emerging as adults from May to early July (Cappaert et al. 2005; Wei et al. 2007; Valenta et al. 2017).

Since its detection in North America in 2002, EAB has spread rapidly, killing hundreds of millions of ash trees across the United States and Canada. Efforts to control EAB spread and mitigate its impact have been challenging and costly (Cappaert et al. 2005; Herms and McCullough 2014). In Europe, the beetle was first detected in Moscow, Russia, in 2003 (EPPO 2007), reported in the Luhansk Oblast province, Ukraine, in 2019 (Drogvalenko et al. 2019; EPPO 2019) and confirmed in Kyiv, Ukraine, in 2023 (EPPO 2023a). The pest continues to spread in both North America, European Russia and Ukraine, maintaining an average annual spread rate of approximately 50 km between 2002 and 2018, accelerated by human activities (Smitley et al. 2008; Webb et al. 2021).

Emerald ash borer is categorized as an A2 pest recommended for regulation as quarantine pests by the European and Mediterranean Plant Protection Organization (EPPO) that poses significant risks to European ash trees (EPPO 2013). Although it has not yet been detected within the EU territory, its westward migration from the most recent findings in western Russia and Ukraine is expected. The risk of introduction increases through hitchhiking, transport on wood packaging, and natural adult flight dispersal (Orlova-Bienkowskaja et al. 2020; Volkovitsh et al. 2021; Meshkova et al. 2023). Systematic monitoring and regulation efforts are therefore crucial to allow an early detection of the pest which is essential for conserving ash populations, which hold ecological and economic significance (EPPO 2013).

Conventional risk management strategies focus on interception and early detection to minimize accidental introductions and mitigate pest damage. These strategies require reliable and cost-effective identification methods. Morphological identification of EAB requires a high level of expertise and can lead to misidentification of other native Buprestidae species. Moreover, morphological identification is limited to intact specimens. On the other hand, DNA-based identification allows the identification of all life stages, including insect-derived environmental samples (Balakrishnan 2005; Scheffer et al. 2006; Floyd et al. 2009; Krehenwinkel et al. 2022; Roger et al. 2022; Kyle et al. 2024). Currently, only a limited number of molecular methods, specifically a Loop-mediated Isothermal Amplification (LAMP) assay developed by Kyei-Poku et al. (2020) and recommended by EPPO, are available to identify EAB. Alternatively, barcoding, involving the amplification of the mitochondrial cytochrome oxidase subunit 1 (COI) (Folmer et al. 1994; Simon et al. 1994), was extensively applied by Kelnarova et al. (2019) to identify more than 100 *Agrilus* species from the Northern Hemisphere, representing roughly 3% of the known *Agrilus* diversity. In a surveillance setting, this approach may, however, encounter challenges due to mixed DNA samples and a lack of proper assessment regarding inclusivity and exclusivity (EPPO 2023b; Kelnarova et al. 2019). The LAMP assay, along with its more recent validation by Peterson et al. (2023a) on European *Agrilus* species, can effectively differentiate EAB from native *Agrilus* species. Although LAMP assays generally offer robust, rapid, and accurate results, Real-Time PCR, also known as quantitative PCR (qPCR), often surpasses their sensitivity in detecting genomic DNA with detection limits in the order of femtograms (Khan et al. 2018; Khodaparast et al. 2024). This has been demonstrated in other assays with a close congener of EAB, *A. anxius* (Peterson et al. 2023b). A key advantage of qPCR is its adaptability. Multiplex qPCRs can be

developed to detect multiple species, a task that is more challenging with LAMP assays (Crego-Vicente et al. 2024). More recently, Kyle et al. (2024) have published a qPCR assay with a detection limit of 1.6 fg gDNA per reaction. This assay was tested against 10 beetles from the North American fauna and focused on eDNA of larvae from vascular tissue.

The objective of the present study was to establish a TaqMan qPCR protocol suitable for the detection of *A. planipennis*, for use in molecular diagnostics and surveillance activities. To achieve this, a PCR based assay (Kyei-Poku et al. 2020) was tailored to a TaqMan Probe assay, and a universal endogenous amplification control (Mittelberger et al. 2020) for eukaryotic DNA samples was incorporated. The assay was optimized and validated for specificity, diagnostic sensitivity, accuracy and robustness using a DNA testing panel focused on European *Agrilus* spp. Subsequently, its performance, and suitability for analysing insect-derived environmental samples, including eggs, egg casings, faeces and frass samples from adults and larvae were evaluated.

Materials and methods

Insect specimens, DNA extraction and species verification

The assay was evaluated by using DNA samples and beetles provided by Peterson et al. (2023a) and that were previously tested with a LAMP-assay for EAB (Suppl. material 1: table S1) and with a LAMP and a qPCR assay for *A. anxius* (Peterson et al. 2023b). In cases where DNA availability was limited or inhibitory compounds were present in the eluate, new DNA was extracted from the provided specimens (Suppl. material 1: table S1). Specimens chosen for extraction were preliminarily washed in molecular grade water (Merck, Darmstadt, Germany). Afterwards, legs or heads, used for DNA extraction, were removed using flame-sterilized forceps and scalpels. These body parts were then put into a 2 ml Eppendorf tubes containing two sterile glass beads (1 mm diameter) and ground using the MM400 bead mill (Retsch) for 2 min at 30 Hz per sec. Post-grinding, DNA extractions were carried out employing the DNeasy Blood and Tissue kit (Qiagen), strictly following the manufacturer's instructions. The DNA was eluted in 100 µl elution buffer and reapplied to the column to enhance DNA yield.

In addition, we complemented our validation panel with DNA (Suppl. material 1: table S1) obtained through our molecular diagnostics activity at WSL conducted to monitor quarantine pests and pathogens in Swiss forests. Samples were sourced from collections made in collaboration with Swiss foresters and cantons from 2012 to 2024. The extraction protocol for these samples involved the use of legs, full bodies, or larvae, which were crushed using MM400 bead mills or mini pestles. DNA extraction was carried out utilizing either the DNeasy Blood and Tissue kit (Qiagen) or the NucleoSpin Tissue XS kit (Macherey-Nagel), following the respective manufacturer's recommendations.

To confirm species assignment of used specimens, the COI barcode region was amplified using the universal primers specific to arthropods LCO1490 and HCO2198 (Folmer et al. 1994) or LepF1 and LepR1 (Hebert et al. 2004) as specified in Suppl. material 1: table S1. PCR reactions were carried out with the GoTaq® G2 Hot Start Master Mix (Promega) in accordance with diagnostic guidelines (EPPO 2021). PCR products of the expected band size (709 bp and

648 bp) were purified using the ExoSAP-IT™ Express PCR Product Cleanup kit (Applied Biosystems). Cycle sequencing was performed using BigDye Terminator v3.1 and purified with the Xterminator Kit (Applied Biosystems). Samples were then sequenced in both forward and reverse directions in-house on a 3500 Series Genetic Analyzer (Applied Biosystems). Sequence chromatograms were visually inspected and edited using sequence analysis software (CLC Main Workbench 24). Consensus sequences were generated by aligning forward and reverse sequences and checked manually for potential errors or ambiguities. Consensus sequences were compared against the all-nucleotide database on BOLD (Ratnasingham and Hebert 2007) using BLAST (Basic Local Alignment Search Tool) to determine species identity. Sequence data generated in this study of specimens not provided by Peterson et al. 2023a have been deposited in Genbank (PV801658 – PV801696) and are summarized in Suppl. material 1: table S1. A sequence alignment and the corresponding phylogenetic tree are available in the supplement (Suppl. material 1: table S2, fig. S1). Unedited chromatograms are available upon request from the corresponding author.

Assay design, primers, and probe synthesis

To design this qPCR assay, we employed the EAB-specific primers EABFOT and EABROT, originally developed by Kyei-Poku et al. (2020). We designed the TaqMan probe EAB-RC-P1 using alignments in CLC Main Workbench 7 (Qiagen) and designed the probe by applying OligoArchitect™ Online (Sigma-Aldrich, http://www.oligoarchitect.com/LoginServlet). Additionally, to ensure the presence of amplifiable DNA, we incorporated the primers UNI28S-fwd and UNI28S-rev and the probe UNI28S-P as internal controls into the assay (Mittelberger et al. 2020).

HPLC-purified Primers (Table 1) were ordered from Microsynth AG (Balgach, Switzerland), while the TaqMan probes EBA-RC-P1 and UNI28S-P (Table 1) were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA) and Eurogentec (Seraing, Belgium), respectively.

qPCR reactions were prepared in a 20 µl volume using the Takyon No ROX Probe Core Kit, including the Takyon enzyme, a modified Taq DNA polymerase designed for faster PCR cycling (Eurogentec) (Table 2) and executed on the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). The PCR protocol included an initial denaturation and enzyme activation step at 95 °C for 3 min, followed by 50 cycles at 95 °C for 10 sec and at 60 °C for 1 min. Results were analysed using the Thermo Fisher Cloud platform with the Design and Analysis New (DA2) and Project apps.

Plasmids (pUC57 derivatives) containing a 229 bp fragment of the mitochondrial COI gene (*Agrilus planipennis*, voucher EAB1; GenBank accession PV801687.1) were synthesized by Eurogentec and used to generate the standard curve. The plasmids were diluted in a tenfold dilution series from 5×10^7 copies/µl to 0.5copies/µl. For the qPCR standard curve, 5 µl from each dilution step was utilized, yielding standard concentrations ranging from 2.5×10^8 to 2.5 copies per reaction (Fig. 1). To determine the limit of quantification, the dilution points of 10, 5, 1, and 0.5 copies were added. A logistic regression model, implemented in RStudio (version 2025.05.0+496), was used to estimate the copy number corresponding to a 95% detection probability. The relationship between copy number and Ct values was evaluated using Spearman's rank correlation coefficient.

Table 1. qPCR primers and probe for *Agrilus planipennis* and internal 28S control. + prior to the nucleotide code indicates that the following nucleotide is a locked nucleic acid (LNA).

Target	Oligo	Sequence	Gene	Length (bp)	Fragment (bp)	Reference
Agrilus planipennis (EAB)	EABFOT	TCAAAGAATGATGTATTTAAGTTTCGATC	COI	29	229	Kyei-Poku et al. 2020
	EABROT	TAGCAATTTTTAGACTTCATTTAGCTGG	COI	28		Kyei-Poku et al. 2020
	EAB-RC-P1	FAM-TATGGTAATTGCTCCCGCAAGAACAGGT-QSY	COI	28		This study
Internal control (UNI)	UNI28S-fwd	CTACTATCTAGCGAAACC	28S	18	84	Mittelberger et al. 2020
	UNI28S-rev	AYTAGAGTCAAGCTCAAC	28S	18		Mittelberger et al. 2020
	UNI28S-P	JOE-AAA+G+A+AG+A+C+C+C+T-BHQ1	28S	12		Mittelberger et al. 2020

Table 2. Components, suppliers, concentrations and volumes for qPCR reaction.

Components	Suppliers	Stock conc.	Reaction conc.	Volume per reaction (µl)
DNA template	–	–	–	5.00
Molecular grade water	Merck	–	–	6.96
Takyon core kit buffer	Eurogentec	10×	1×	2.00
Takyon core kit $MgCl_2$	Eurogentec	50 mM	5.5 mM	2.20
dNTPs	Eurogentec	5 mM	0.4 mM	1.60
ROX Reference Dye	Invitrogen	50×	0.1×	0.04
EABROT	Microsynth	10 µM	0.3 µM	0.60
EABFOT	Microsynth	10 µM	0.3 µM	0.60
EAB-RC-P1	Applied Biosystems	10 µM	0.1 µM	0.20
UNI28S-fwd	Microsynth	10 µM	0.15 µM	0.30
UNI28S-rev	Microsynth	10 µM	0.15 µM	0.30
UNI28S-P	Eurogentec	10 µM	0.05 µM	0.10
Takyon Enzyme	Eurogentec	5 U/µl	0.5 U	0.10
Final reaction volume				20.00

Specificity and sensitivity

To ensure DNA quality and rule out false negatives, DNA was considered amplifiable if the 28S Ct value was ≤ 32 (Suppl. material 1: table S2). To test the specificity of the main assay, primers were evaluated against a validation panel consisting of European *Agrilus* spp. and other woodboring beetles (Suppl. material 1: table S1). DNA from this panel was diluted 1:10 with molecular-grade water (Merck), and 5 µl of diluted DNA was used per qPCR reaction. Samples were considered positive if their Ct values were lower than the lowest dilution point of the standard curve (2.5 copies per reaction). To assess sensitivity, DNA concentrations of three *A. planipennis* (EAB) samples (EAB1, EAB2, EAB3) were quantified using the Qubit dsDNA Broad Range Kit (Invitrogen) on a Qubit 3.0 fluorometer. Each sample was serially diluted in tenfold steps, ranging from 1 ng/µl to 1 fg/µl. Triplicate qPCRs were performed for each dilution to determine the limit of detection (LOD). A logistic regression model implemented in RStudio was used to estimate the DNA concentration corresponding to a 95% detection probability.

Frass, egg and faeces samples

Fresh EAB samples were obtained from an experimental setup within the biosafety level 3 facilities of WSL (Gossner et al. 2023). EAB eggs were sourced from two colonies maintained at the Great Lakes Forestry Centre, originally initiated from adults flushed from green ash (*Fraxinus pennsylvanica*) bolts collected in Brighton, ON, Canada (Roe et al. 2018).

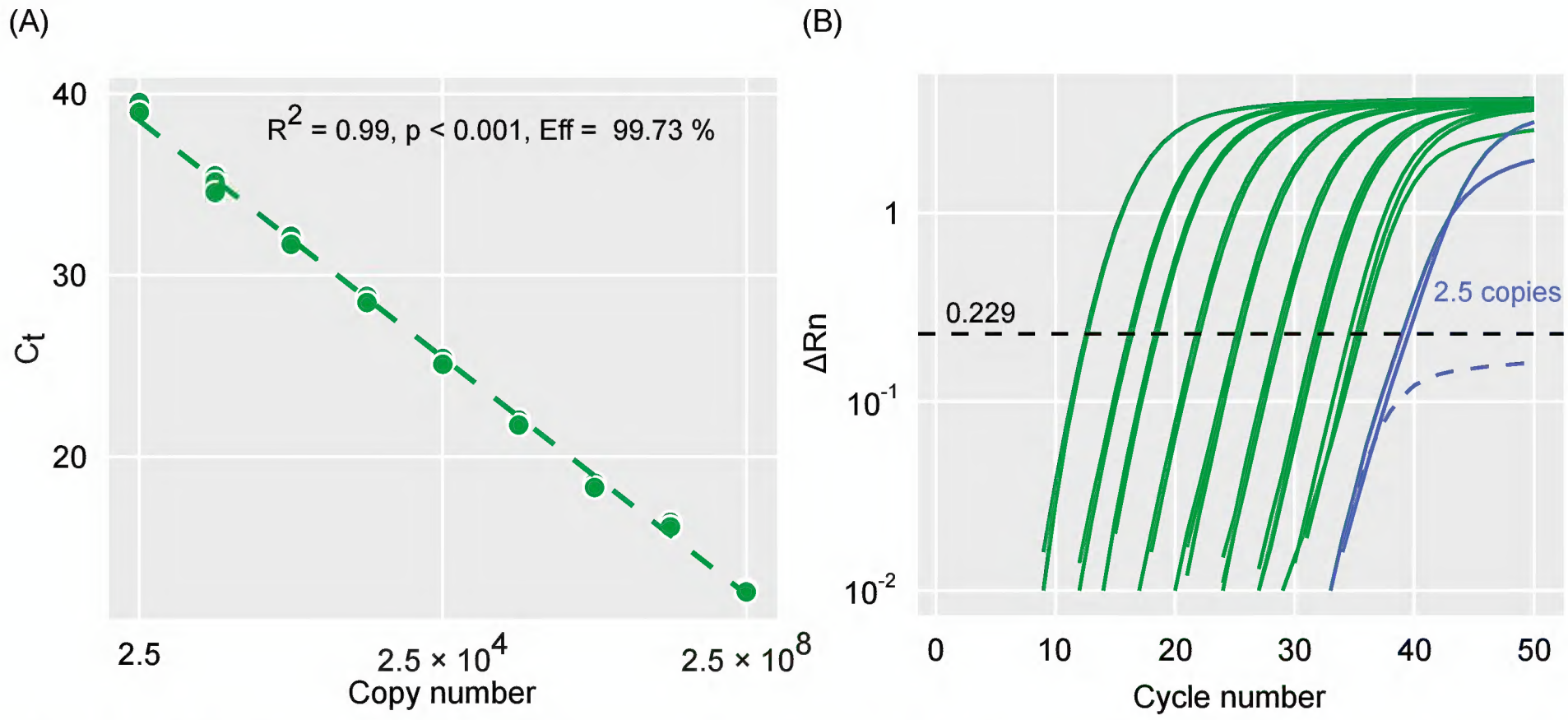


Figure 1. **A** Standard curve of the qPCR EAB assay, generated using a pUC57-derived plasmid containing a 229 bp fragment of the mitochondrial COI gene corresponding to the reference sequence EAB1 (PV801687). Target copy number was plotted against Ct values, demonstrating strong linearity across the dilution range from 2.5 to 2.5×10^8 copies per reaction, with an R^2 value of 0.999. Copy numbers are shown on a logarithmic scale. The calculated assay efficiency was 99.73% **B** Amplification curves from qPCR reactions performed in triplicate across a tenfold dilution series ranging from 2.5×10^8 to 2.5 copies. ΔRn values are plotted on a logarithmic scale. The threshold (0.229), indicated by a dashed line, was automatically determined using QuantStudio's default settings. The lowest dilution, containing 2.5 copies and shown in blue, falls below the calculated detection limit of 2.7 copies.

Larvae, eggs, egg casings, frass samples of larvae and adults as well as faeces of adult beetles were used to validate the usability of the assay for environmental samples (Table 3). For the larval woody frass samples, we collected frass from EAB larval galleries in potted European ash trees (*Fraxinus excelsior*). For adult EAB frass samples, one *F. excelsior* leaf was added to a closed cylinder containing one to six adult beetles. Leaves were in contact with beetles for 24, 48 or 72 hours, after which they were immediately frozen at –20 °C in a sterile 50 ml falcon tube. Eggs, eggshells and larvae were ground in a bead mill (MM400, Retsch) using two 1.4 mm ceramic beads for 1 min at 30 Hz and then extracted with the NucleoSpin Kit (Macherey-Nagel) following manufacturers recommendations and eluted in 25 µl. Larval frass and adult faeces samples were ground using one 3 mm steel bead for 1 min at 30 Hz, extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following manufacturers recommendations and eluted in 100 µl. Adult frass samples were lyophilized overnight, ground in a bead mill (MM400) using two 3 mm steel beads for 2 min at 30 Hz, extracted using the DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's recommendations and eluted in 100 µl. DNA extracts were diluted 1:10, 1:20, 1:100 and 1:200 and qPCRs performed with 5 µl of each dilution as well as undiluted DNA.

Blind test

A total of 10 DNA samples suspected to contain *A. planipennis* DNA were sent on dry ice to two different diagnostic labs (CNR – IPSP, IT & Agroscope, CH) and two research labs in Switzerland unrelated to forest health or diagnostics (Aquatic Ecology, EAWAG & Plant and Microbial Biology, UZH). Primer pairs and a probe

Table 3. Environmental samples for assay validation.

Sample type	Extraction kit	Quantity	DNA dilutions
Larvae	MN Tissue XS	1–4 individuals	1:10
Eggs	MN Tissue XS	2 eggs	1:10
Egg casings	MN Tissue XS	5 & 12 pieces	1:10
Faeces	QIAGEN Blood&Tissue	5–50 mg 1–15 pieces	undiluted, 1:10, 1:20, 1:100, 1:200
Larval frass (bore dust)	QIAGEN Blood&Tissue	5–60 mg	undiluted, 1:10, 1:20, 1:100
Adult frass (leaves)	QIAGEN Plant Mini	20 mg	undiluted, 1:10, 1:20, 1:100, 1:200

to detect *A. planipennis* (EAB), primer pairs and a probe targeting the 28S region as an amplification control, and the plasmid construct to establish the standard curve were included. The samples and no-template controls were anonymized and assigned random codes to ensure blinding of the testing personnel. The researchers conducting the blind test were unaware of the true identities of the samples during the analysis. A list of the samples can be found in Fig. 3.

Each sample was processed according to the established protocol for qPCR analysis. The qPCR reactions were performed in duplicate or triplicate to ensure the reliability and accuracy of the results. The qPCR reaction kit and instruments were chosen according to the specifications of the respective laboratories.

Results

Molecular identification of specimens

The identity of specimens used in the validation panel could be confirmed by sequencing the COI barcode (Suppl. material 1: table S1). Only for the species *Aegomorphus clavipes* (Cerambycidae) no barcode sequence could be generated.

Assay design and efficiency

The plasmid construct containing the synthesized COI amplicon from *A. planipennis* was diluted in tenfold steps. For the qPCR standard curve, the standard concentrations ranged from 2.5×10^8 to 2.5 copies per reaction. Plots of Ct values versus $\log_{10}$ copy number indicated that the reaction efficiency of the target amplicon was above 99%, with an R^2 value exceeding 0.99 (Fig. 1A). The amplification plot showed curves with a clearly distinguishable exponential phase followed by a plateau phase (Fig. 1B).

Specificity of multiplex TaqMan PCR

The specificity of the qPCR assay was evaluated using a validation panel comprising adults from various geographic origins and larvae (Table 1) to assess inclusivity, and relevant non-target species to evaluate exclusivity. The assay was specific to *Agrilus planipennis* and no other tested species showed amplification (Suppl. material 1: table S2). For the species *Agrilus betuleti* late amplification of the internal control (28S) was observed, likely due to bad DNA quality.

Sensitivity of multiplex TaqMan PCR

The limit of quantification (LOQ) of the assay was evaluated using serial dilutions of plasmid DNA. The LOQ was determined to be 2.7copies per reaction at probability of 95%. The limit of detection (LOD) was assessed using serial dilutions of *A. planipennis* genomic DNA extracted from adult specimens, ranging from 1 ng/µl to 1 fg/µl. The assay consistently detected as little as 0.5 pg of DNA per reaction for each specimen tested. Amplification curves showed distinct sigmoidal patterns even at these minimal DNA concentrations, indicating the assay's sensitivity to reliably detect *A. planipennis* DNA at low levels (Fig. 2). Based on a logistic regression model applied to dilution series from three specimens, the LOD was estimated at 0.403 pg (Fig. 2D).

Selectivity

To gauge the performance of the qPCR assay in detecting environmental samples, an assessment was conducted to analyse performance variations based on the matrix (Table 3). Larvae, egg and egg casing samples produced strong amplifications for both the EAB specific probe as well as the 28S internal control. Faeces samples showed strong inhibition during the PCR so a DNA-dilution of 1:20 was necessary. In 5–20 mg of faeces, EAB DNA was reliably detectable (Ct < 29). For higher faeces amounts (50 mg), a DNA-dilution of 1:200 was necessary due to inhibition. In a single faecal pellet EAB DNA could be detected with a Ct value of 35.7. In larval frass samples from galleries (bore dust), PCR inhibition was an issue, requiring a DNA-dilution of 1:100. With this dilution, EAB DNA could be reliably detected in 20 mg of frass. In lower amounts detection was possible but no longer reliable. For adult frass samples (leaves), EAB DNA could be detected in 20 mg of leaf powder that was in contact with adult beetles (1–6) for 48 or 72 hours. All the positive amplifications for these adult frass samples had a higher Ct value than the lowest standard dilution (25 copies).

Blind test validation of the qPCR method for the detection of *Agrilus planipennis*

To assess the robustness and reproducibility of the developed qPCR method for the detection of *Agrilus planipennis*, a blind test was conducted. All labs participating in the blind test were able to accurately identify the samples containing DNA of *A. planipennis*. There were no false positive or false negative reactions observed for EAB.

The implemented amplification control, targeting the 28S region was included in the blind test to validate the detection of amplifiable DNA. Lab #1 and #4 showed amplifiable DNA for all samples, Lab #3 had a single sample with no internal control amplification, while Lab #2 was not able to amplify any DNA using the 28S amplification control. Negative control samples, devoid of *A. planipennis* DNA (NTC), were also included to monitor for potential contamination. No amplification was observed in the no-template controls (NTCs), confirming the absence of contamination during the PCR setup. The results of the blind test are summarised in Fig. 3.

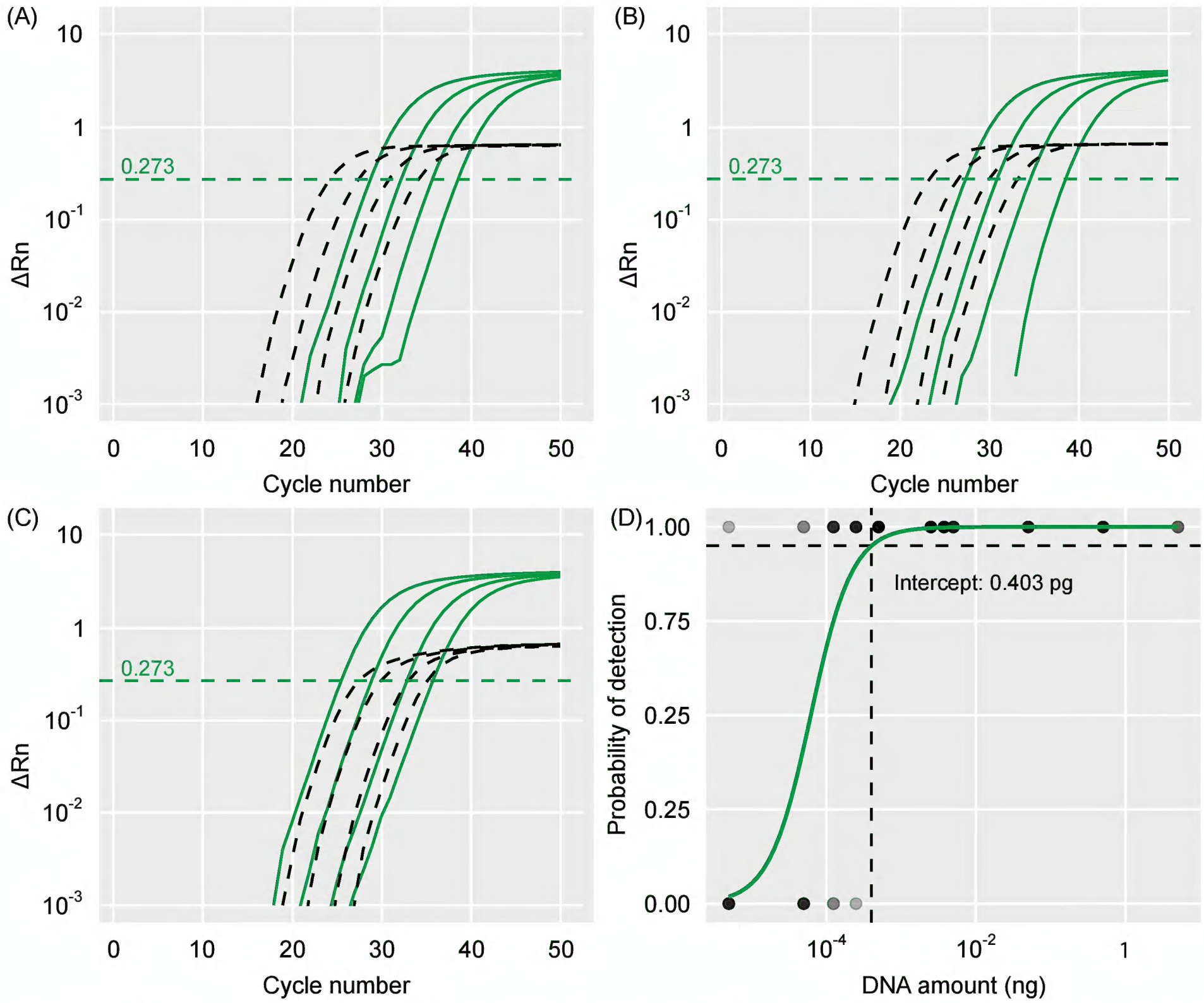


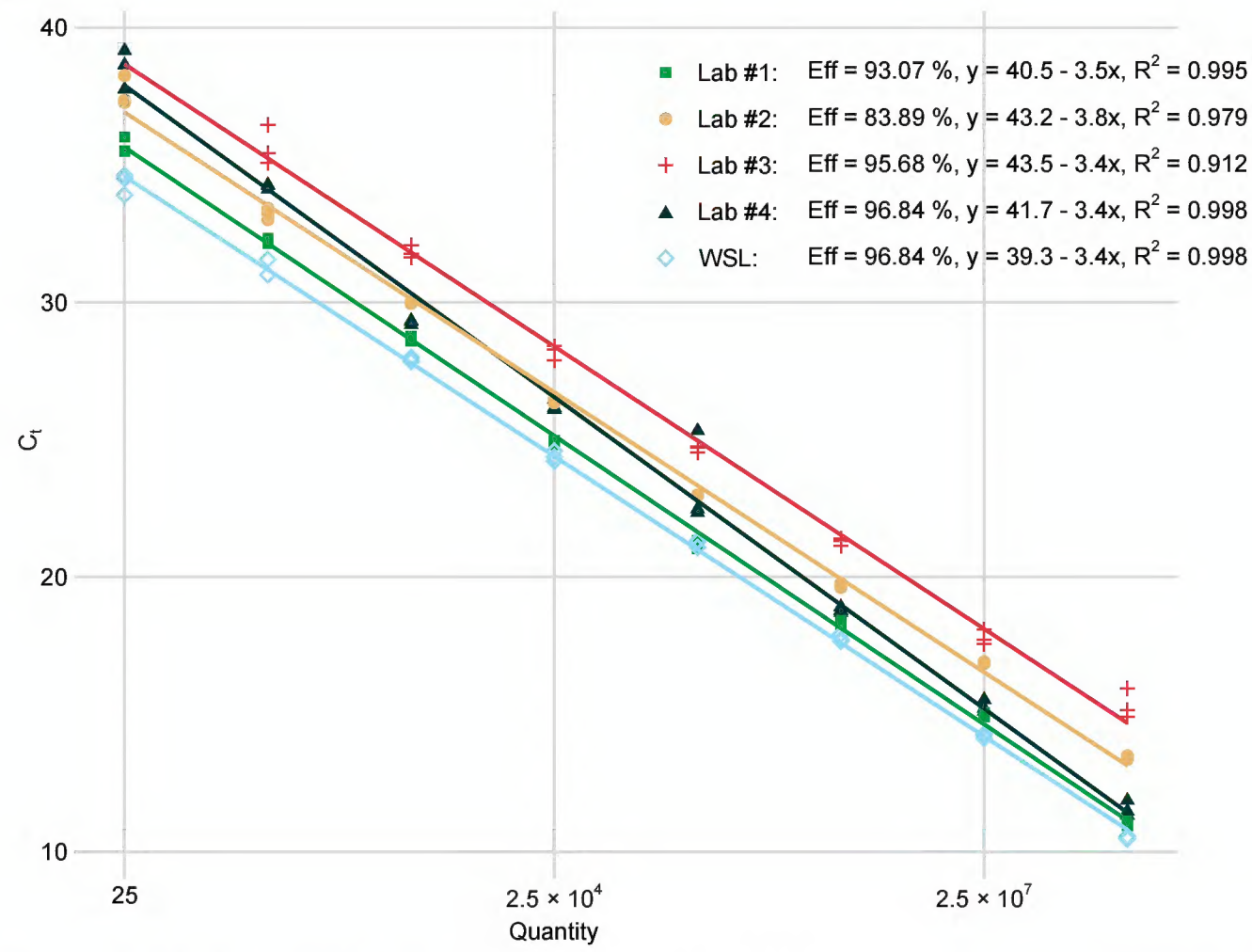
Figure 2. Amplification curves are shown for tenfold dilution series of genomic DNA (0.5 ng to 0.5 pg per reaction; green) from specimens EAB1, EAB2, and EAB3 (A, B, and C). The threshold for target detection (0.273), indicated by a horizontal dashed line, was set automatically in QuantStudio. Black dashed lines represent amplification of the internal 28S control. (D) A logistic regression curve was used to determine the limit of detection (LOD), with the intercept corresponding to a 95% detection probability. The logistic regression was applied to dilution series ranging from 5 ng to 5 fg of genomic DNA from the same three specimens, with each dilution tested in 3–6 replicates.

Discussion

For a correct identification of *Agrilus planipennis* EPPO currently recommends to use morphological identification, DNA-Barcoding and a LAMP assay (EPPO 2023b). The qPCR assay presented here complements these methods as an additional accurate, quick, and comparatively affordable approach. By converting the existing LAMP assay (Kyei-Poku et al. 2020; Peterson et al. 2023a) into a TaqMan Probe assay and incorporating a universal internal amplification control (28S rDNA), the assay achieves a high detection capability and reliability across a wide range of sample types, including environmental matrices.

The assay demonstrated high specificity, successfully distinguishing *A. planipennis* from a broad panel of European *Agrilus* species and other non-target taxa relevant to forestry. The testing panel included closely related buprestid beetles, which are often morphologically similar and that will co-occur with EAB in European

(A)



(B)

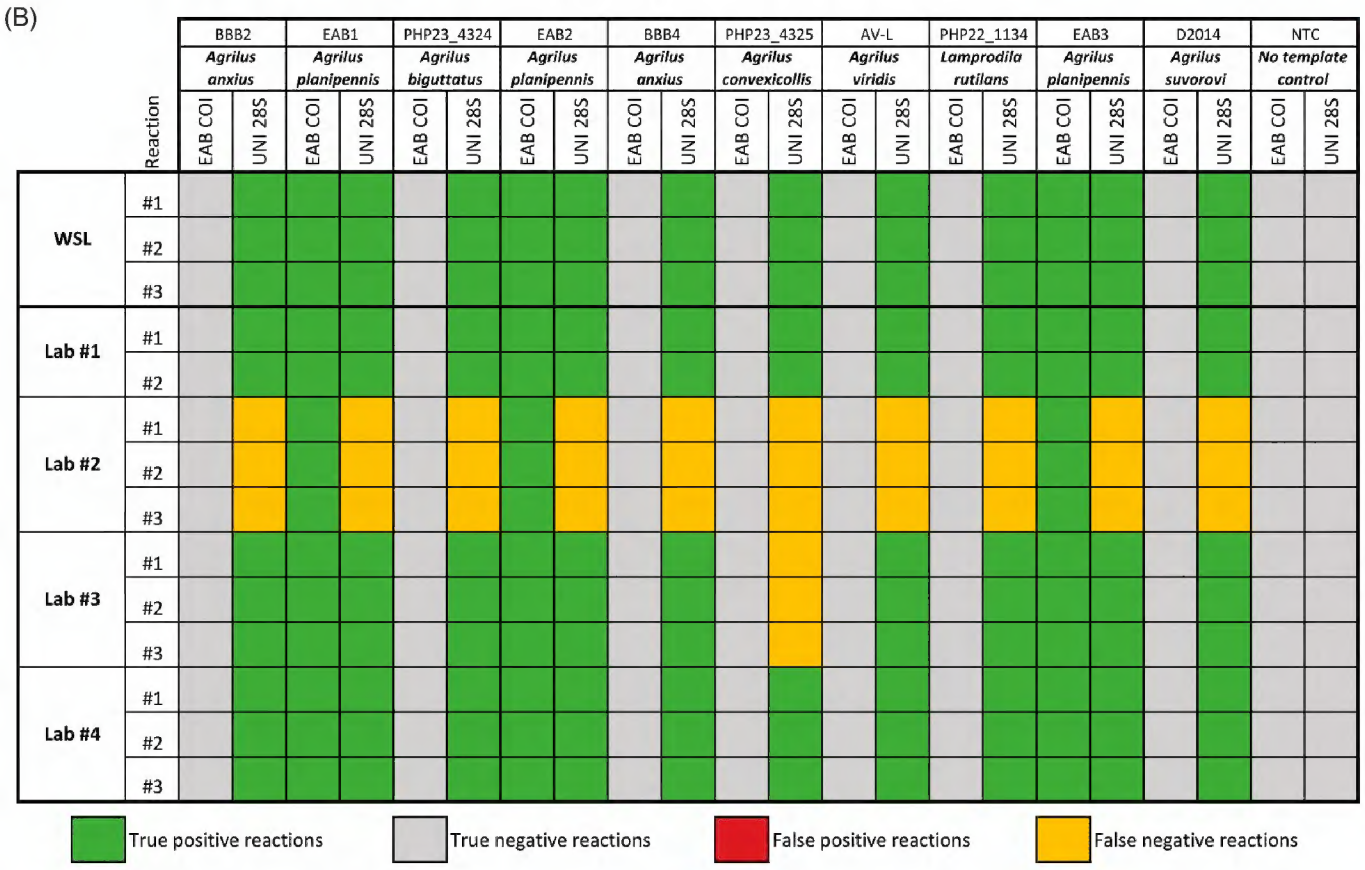


Figure 3. Results of the blind test. Sample names were anonymized (EAB_B01–EAB_B10) including the no-template control (NTC) **A** Standard curves generated by individual laboratories using COI-specific oligonucleotides and the plasmid construct. For each laboratory, PCR efficiency, the corresponding linear regression equation, and the coefficient of determination (R^2) are provided **B** qPCR reagents and instruments used by each lab: **Lab 1:** Promega GoTaq Probe qPCR Master Mix (A6102) on a Bio-Rad CFX Opus 96. **Lab 2:** Applied Biosystems TaqMan Universal PCR Master Mix (4304437) on a StepOnePlus System. **Lab 3:** KAPA PROBE FAST qPCR Master Mix (KK4701) on a Bio-Rad CFX Opus 96. **Lab 4:** Promega GoTaq Probe qPCR Master Mix (A6102) on a QuantStudio 3.

forests. No cross-reactivity was observed, confirming the taxonomic resolution of the assay. The late amplification of the internal control in *Agrilus betuleti* is more likely due to poor DNA quality, than to non-specific binding.

The limit of detection, regarding sensitivity, was established at 0.4 pg of *A. planipennis* genomic DNA per reaction. These results highlight the suitability of the assay for low-template samples, a common challenge in early detection and

monitoring programs. Compared to other molecular techniques, such as LAMP (Kyei-Poku et al. 2020; Peterson et al. 2023a), this qPCR assay offers similar sensitivity while maintaining rapid and specific detection, making it suitable for implementation in routine diagnostic workflows.

The applicability of the assay to environmental samples, such as eggshells, frass, and faeces, demonstrates its practical value in surveillance. Despite PCR inhibition, commonly associated with complex matrices like faeces and frass, appropriate DNA dilutions restored amplification efficiency. Notably, even a single faecal pellet yielded detectable EAB DNA, confirming the assay's potential in early detection strategies. This is especially relevant for passive sampling in high-risk areas where the presence of adult beetles or larvae is uncertain. We envision the application of this assay both in early detection monitoring of EAB and in routine diagnostic tasks, particularly in interception scenarios where morphological identification is hindered due to specimen integrity or the presence of frass samples only. In terms of field applicability, wet trapping remains a widely used monitoring technique. Current investigations within our institution are exploring the analysis of trapping liquids to detect potential DNA of quarantine organisms. A robust molecular assay could enhance the diagnostic workflow by significantly reducing the time needed for morphological identification. Overall, our assay is designed to serve as a reliable alternative for EAB identification and complementary to existing diagnostic tools.

In blind tests conducted across independent diagnostic and research laboratories, the assay showed consistent performance and transferability, with accurate detection of *A. planipennis* DNA. This inter-laboratory validation underscores the robustness of the assay protocol and its suitability for integration into broader surveillance networks. However, the failure to amplify the internal control in a single sample by two laboratories, and its absence across all samples in one laboratory, indicates a lack of robustness in the internal control across different laboratories and platforms. To ensure reliable molecular diagnostics, the inclusion of an external positive amplification control is recommended in such cases.

Taken together, the developed qPCR assay fulfils critical criteria for molecular diagnostic tools recommended by EPPO and other phytosanitary organisations focusing on specificity, sensitivity, reproducibility, and applicability across diverse sample types. Given the rapid and continued westward spread of EAB in Europe and the increasing risk of accidental introduction into EU territory, the implementation of such assays will play a pivotal role in early detection, rapid response, and containment strategies. Future efforts may include further multiplexing capabilities for simultaneous detection of multiple invasive *Agrilus* species.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

BR and QK conceptualized the study. DLP, APG and DH organised the samples. SS designed the EAB probe. Lab work was performed by QK, LCF and DLP. FP, DA, MG and HZ performed the blind tests. QK and BR visualized the data and wrote the manuscript. QK and BR finalized the manuscript with comments from the other co-authors.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Additional information

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Data type: docx

Explanation note: **table S1.** Insect species used for specificity testing of EAB qPCR primers, including geographic origin, sample type, extraction method and GenBank accession number of COI sequences. **table S2.** qPCR results and Ct-values for internal control for all samples from the validation panel. With an internal control Ct-value of 32 or lower DNA is considered amplifiable. **fig. S1.** Sequence alignments of the COI region from Agrilus planipennis and non-target species included in this assay. **fig. S2.** Sequence phylogeny of target and nontarget species tested in this assay.

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